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Species differences in methanol and formic acid pharmacokinetics in mice, rabbits and primates ☆

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ABSTRACT

Methanol (MeOH) is metabolized primarily by alcohol dehydrogenase in humans, but by catalase in rodents, with species variations in the pharmacokinetics of its formic acid (FA) metabolite. The teratogenic potential of MeOH in humans is unknown, and its teratogenicity in rodents may not accurately reflect human developmental risk due to differential species metabolism, as for some other teratogens. To determine if human MeOH metabolism might be better reflected in rabbits than rodents, the plasma pharmacokinetics of MeOH and FA were compared in male CD-1 mice, New Zealand white rabbits and cynomolgus monkeys over time (24, 48 and 6 h, respectively) following a single intraperitoneal injection of 0.5 or 2 g/kg MeOH or its saline vehicle. Following the high dose, MeOH exhibited saturated elimination kinetics in all 3 species, with similar peak concentrations and a 2.5-fold higher clearance in mice than rabbits. FA accumulation within 6 h in primates was 5-fold and 43-fold higher than in rabbits and mice respectively, with accumulation being 10-fold higher in rabbits than mice. Over 48 h, FA accumulation was nearly 5-fold higher in rabbits than mice. Low-dose MeOH in mice and rabbits resulted in similarly saturated MeOH elimination in both species, but with approximately 2-fold higher clearance rates in mice. FA accumulation was 3.8-fold higher in rabbits than mice. Rabbits more closely than mice reflected primates for *in vivo* MeOH metabolism, and particularly FA accumulation, suggesting that developmental studies in rabbits may be useful for assessing potential human teratological risk.

Introduction

Human exposure to methanol (MeOH) can result from its use as an industrial solvent, through accidental ingestion and as a potential alternate fuel source (Harris et al., 2004). Most toxicological studies use rodents as models of human MeOH toxicity, but there are key species differences in MeOH metabolism. Primates, including humans, metabolize MeOH to formaldehyde using the enzyme alcohol dehydrogenase (ADH), whereas rodents use catalase (Cederbaum and Qureshi, 1982) (Fig. 1). Humans and rodents metabolize formaldehyde to formic acid (FA) by formaldehyde dehydrogenase (ADH III). FA is subsequently converted to the non-toxic metabolites carbon dioxide and water by a folate-dependent dehydrogenase (Johlin et al., 1987). Humans have limited folate, resulting in FA accumulation following higher MeOH exposures (Perkins et al., 1995). Conversely, folate is not limited in rodents, which metabolize FA via both catalase- and folate-dependent pathways, thereby preventing FA accumulation (Clary, 2003; Harris et al., 2004). Aside from its role in rodent MeOH metabolism, catalase in all species provides cytoprotection against reactive oxygen species (ROS) by detoxifying hydrogen peroxide (Halliwell and Gutteridge, 2007; Wells et al., 2009), which complicates the interpretation of rodent data for MeOH toxicity.

Human MeOH overdose causes acute ocular toxicity, CNS depression and death, apparently due to FA accumulation and subsequent metabolic acidosis (Lanigan, 2001; Wallage and Watterson, 2008). Rodents are resistant to this acute toxicity, presumably due to the absence of FA accumulation. Conversely, rodents can be susceptible to delayed adverse consequences of MeOH exposure, such as fetal neural tube defects and cleft palates following *in utero* exposure (Bolon et al., 1994; Rogers and Mole, 1997; Rogers et al., 2004), and possibly cancer in adult rats (Soffritti et al., 2007), although the latter remains controversial (Cruzan, 2009; Schoeb et al., 2009). Mechanisms of acute MeOH toxicity likely differ from those underlying its delayed toxicities. Although the mechanisms underlying delayed MeOH toxicities are

Abbreviations: ADH, alcohol dehydrogenase; ADH III, formaldehyde dehydrogenase; AO, alcohol oxidase; AUC, area under the plasma concentration–time curve; Cl, clearance; FA, formic acid; FDH, formate dehydrogenase; FID, flame ionization detector; GC, gas chromatography; MeOH, methanol; NZW, New Zealand white; ROS, reactive oxygen species; SOD, superoxide dismutase.

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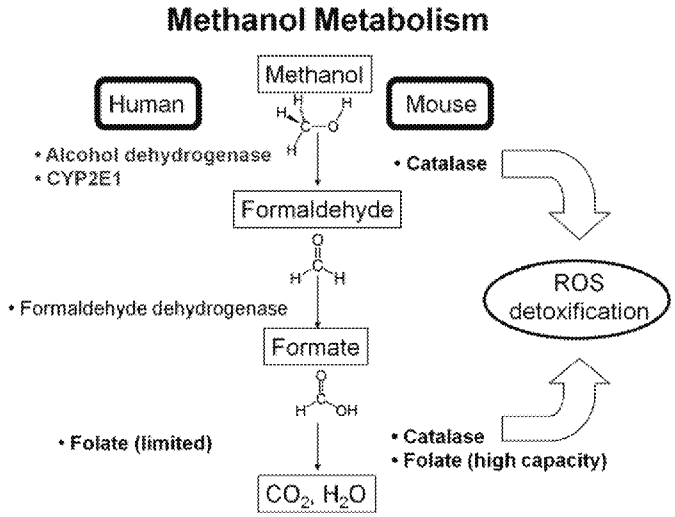

Fig. 1. Species differences in the enzymes catalyzing the metabolism of methanol (MeOH) to formaldehyde and formic acid (FA) in mice and primates, including humans. In addition to its role in MeOH metabolism in mice, catalase is used by all species in the detoxification of reactive oxygen species (ROS).

unclear, a role for ROS has been implicated in a rat embryo culture study, where depletion of the antioxidative peptide glutathione (GSH) increased the embryopathic effect of MeOH and its metabolites (Harris s et al., 2004). The proximal toxicant of MeOH developmental toxicity is unknown, although in rat embryo culture exogenous administration of formaldehyde is substantially more embryopathic than MeOH and FA (Harris et al., 2004; Hansen et al., 2005). Direct *in vitro* exposure to formaldehyde, which is highly reactive and transient, may not accurately reflect *in utero* exposure via its production from MeOH metabolism, but a role for formaldehyde in MeOH developmental toxicity remains plausible. ROS also have been implicated in adult MeOH toxicity, including *in vivo* rat studies reporting that MeOH increased lipid peroxidation in lymphoid organs and the brain, as well as decreasing GSH and activity of the antioxidative enzyme superoxide dismutase (SOD) (Farbiszewski et al., 2000; Parthasarathy et al., 2006).

The species differences between rodents and humans in MeOH metabolism and susceptibility to at least acute toxicity suggest that rodents may not constitute the most predictive model of human risk for delayed adverse effects of MeOH. Conversely, the rabbit is an attractive non-primate alternative for several reasons. Rabbits, but not mice, are susceptible to enhanced embryonic oxidative DNA damage and teratogenic effects of thalidomide, a known ROS-initiating drug, which may prove relevant to the delayed effects of MeOH (Fratta et al., 1965; Parman et al., 1999). Additionally, rabbits but not rats are insensitive to ethylene glycol teratogenicity, where the toxicokinetic profile in rabbits more closely reflects that in humans (Carney et al., 2008). Finally, there is *in vitro* evidence in liver homogenates to suggest that ADH activity in alcohol metabolism is more similar in rabbits than mice to that in humans (Otani, 1978).

To determine if primate metabolism of MeOH, and particularly FA accumulation, was reflected more accurately in rabbits than rodents, we examined the *in vivo* plasma pharmacokinetics of MeOH and FA in cynomolgus monkeys, rabbits and mice following single doses of MeOH either below or above the reported saturation level for catalase (600 mg/kg) (NEDO, 1986, 1987). Males were used to avoid potential confounding effects of hormonal differences on MeOH metabolism between animals and species. Our results provide the first *in vivo* evidence that rabbits more closely than mice reflect primate MeOH metabolism, particularly in regard to FA accumulation. Developmental studies are needed to determine if rabbits similarly predict more accurately than mice the human risk for delayed adverse MeOH effects.

Methods

Chemicals. HPLC grade MeOH was purchased from EMD Sereno Canada, Inc. (Mississauga, ON). Saline (0.9%, sterile) was purchased from Baxter Corporation (Mississauga, ON). Isoflurane was purchased from Abbott Laboratories Ltd. (Saint-Laurant, QC). Compressed oxygen (99%) was purchased from BOC Gases (Mississauga, ON). Alcohol oxidase (AO; A6941) and formate dehydrogenase (FDH; F8649) from *Candida boidinii*, diaphorase from *Clostridium kluyveri* (D5540), sodium formate, and β-nicotinamide adenine dinucleotide (β-NAD^+; N1511) were purchased from Sigma-Aldrich (St. Louis, MO). Formaldehyde dehydrogenase from *Pseudomonas putida* was obtained from MP Biomedicals (Solon, OH). *p*-iodonitrotetrazolium violet (INT) was purchased from Alfa Aesar (Ward Hill, MA).

Animals and diet. All animal protocols used were approved by the institutional animal care committee in conformance with the guidelines established by the Canadian Council on Animal Care.

Mice. Male CD-1 mice were purchased from Charles River Laboratories (Saint-Constant, QC) and were 2–5 months old and approximately 33–52 g at the time of study. Mice were housed in vented cages from Allentown, Inc. (Allentown, NJ) with ground corn cob bedding (Bed-O' Cobs Laboratory Animal Bedding, The Andersons Industrial Products Group, Maumee, OH). Animal rooms were climate- and light-controlled (20 °C, 50% humidity, 14-hour light–10-hour dark cycle). Mice were fed rodent chow (Harlan Labs: 2018, Harlan Teklad, Montreal, QC) and provided with water *ad libitum*. All mice were transported from the animal facility to the main laboratory for dosing and euthanizing, but were allowed 1 h for acclimatization prior to the commencement of each study.

Rabbits. Male New Zealand white (NZW) rabbits were purchased from Charles River Laboratories. At the time of experiments, rabbits were between the ages of 5 and 12 months, with a weight range of 3.25–4.75 kg. Rabbits were housed in plastic cages (Allentown, Inc.) in rooms maintained at 20 °C and 60% humidity, with an automated 12-hour light–dark cycle. Rabbits were fed a diet of standard high-fibre rabbit chow (Lab Diet: 5236 Hi-Fibre, PMI Nutrition International LLC, Brentwood, MO), and provided with water *ad libitum*. Three days prior to the commencement of each study, rabbits did not receive any vegetable supplementation to their diet to avoid exposure to exogenous sources of antioxidants.

Primates. Studies were conducted with male cynomolgus monkeys (*Macaca fascicularis*) at Charles River Laboratories (Sparks, NV). At the time of experiments, monkeys were between the ages of 3.4 and 5.7 years, with a weight range of 2.8–4.8 kg. Monkeys were acclimatized to individual stainless-steel cages two weeks prior to commencement of the study in rooms maintained between 18 and 29 °C, with an automated 12-hour light–dark cycle. Monkeys were fed a certified primate chow diet (# 5048) from Purina Mills (St. Louis, MO) supplemented with fruit or vegetables 2–3 times weekly, and provided with water *ad libitum*.

Dosing and blood collection. Mice were administered either a single low dose (0.5 g/kg bw) or high dose (2 g/kg bw) of MeOH (20% [w/v] in sterile saline) or a saline vehicle control. MeOH was administered via intraperitoneal (ip) injection using a 26 gauge (G) 3/8 needle. Prior to blood collection, mice were anaesthetized by placing a 15 mL conical tube containing isoflurane-soaked gauze over the snout of the animal until it was unresponsive. Blood samples (approximately 1 mL) were collected in heparinised vacutainers (lithium heparin 68 USP units per tube, Becton, Dickinson and Company, Oakville, ON) either directly from the atrium or by puncturing the portal vein or inferior vena cava, using a 20 G 1 1/2 needle. For mice administered a

high dose of MeOH, blood samples were collected at 1, 4, 8, 12, 16, 20 and 24 h post injection, while samples for the low dose mice were collected at 1, 2, 4, 6, 8, 10 and 12 h post injection. Plasma was isolated from the samples by centrifugation at 1000×g for 15 min at 4 °C. Plasma samples were frozen at −20 °C until time of analysis. At each time point, plasma samples were collected from 3 to 6 mice.

Due to the size of the animals, rabbits were anaesthetized with 3% isoflurane in 2 L of oxygen for approximately 5 min. Rabbits were then administered a single low or high dose (0.5 or 2 g/kg bw) of MeOH (20% [w/v] in sterile saline) or its saline vehicle (control) by ip injection using a 23 G needle. Following injection, rabbits were exposed to 100% oxygen for approximately 2 min to quicken recovery from the anaesthesia. Blood samples (1–3 mL) were collected in heparinised vacutainers (lithium heparin 68 USP units per tube, Becton, Dickinson and Company) through the ear vein prior to dosing and at 15 and 30 min, and 1, 4, 6, 12, 18, 24, 30, 36 and 48 h post injection. Blood samples were centrifuged at 1000×g for 15 min at 4 °C, and the plasma supernatant was collected and frozen at −80 °C. A total of 3 rabbits were used in each treatment group.

Primates were lightly sedated to effect with ketamine (~5–10 mg/kg im) and then administered MeOH (2 g/kg bw; 20% [w/v] in sterile saline) or a saline vehicle control by ip injection using a 22 G needle. Blood samples (6 mL) were drawn from the femoral vein and collected in heparinised tubes at 15 and 30 min, and 1, 4 and 6 h post injection. Whole blood was processed to plasma by centrifugation at 1500×g for 15 min at 4 °C, flash-frozen in liquid N_2 and stored at −70 °C. Three primates were used in both the 2 g/kg MeOH and saline control groups.

Analytical methods. Plasma samples from saline controls and MeOH-treated rabbits and mice were analyzed for MeOH and FA concentrations by headspace gas chromatography (GC) based on previously published methods (Fraser and MacNeil, 1989; Porter and Moyer, 1994). The Agilent GC system consisted of a 6890N GC, a G1888 headspace sampler, and a G1540N flame ionization detector (FID) coupled with a Restek Rtx-200 capillary column (30 m, 0.53 mm ID, 3 μm). MeOH concentrations were quantified from linear standard curves using the peak area ratios of MeOH to the internal standard n-propanol using Chemstation software (version B.02.01; Agilent). Similarly, FA concentrations were determined based on the peak area ratios of methyl formate to the internal standard methyl propionate.

Due to local biosafety restrictions, MeOH and FA concentrations were analyzed in primate serum samples with validated enzymatic methods. MeOH concentrations were determined by the method of Vinet (Vinet, 1987) adapted to accommodate analysis in a microplate format. Briefly, reaction mixtures contained 200 μL of enzymatic reagent (2.5 mM β-NAD^+, and 500 U/L FDH in 100 mM phosphate buffer, pH 7.6), and 3.5 μL of sample or calibration standards. Reactions were incubated for 5 min at 30 °C and then started by addition of 16.5 μL of AO solution (5 U/mL in 100 mM phosphate buffer, pH 7.6). Calibration standards of 12.5, 6.25, 3.125 and 0 mmol/L were prepared by diluting a 1.25 mol/L aqueous solution of MeOH with unexposed primate serum. Serum samples from MeOH-exposed primates were diluted in unexposed primate serum in order to fit within the calibration curve. Absorbance was monitored continuously at 340 nm in a Spectramax M2 Plate Reader (Molecular Devices Corporation, Sunnyvale, CA) and the initial linear reaction velocities were used to calculate a standard curve to determine unknown concentrations, which were determined from the average of duplicate determinations. Serum FA concentrations were determined by a modification of the colorimetric endpoint assay (Grady and Osterloh, 1986) which was developed to improve the dynamic range of the fluorometric method of Makar and Tephly (1982) using acetonitrile serum extraction and INT, a chromophore with higher molar absorptivity. Briefly, reaction mixtures contained 600 μL enzymatic reagent (2.5 mM β-NAD^+, 800 U/L diaphorase, 2.0 mmol/L INT in 100 mM phosphate buffer, pH 6.0), and 20 μL of supernatant from an acetonitrile (1:1) serum precipitation. Reactions were initiated by the addition of 12 μL of FDH (5400 U/L in 100 mM phosphate buffer, pH 6.0 containing 2.5 mM β-NAD^+, and 800 U/L diaphorase), left for 15 min at room temperature and then the absorbance was read at 500 nm in a Spectramax M2 Plate Reader (Molecular Devices Corporation). Serum FA concentrations were calculated from the average of duplicate determinations using a six point standard curve prepared from 2-fold serial dilutions of unexposed primate serum spiked with 320 mg/ml of sodium formate.

Data analysis. MeOH and FA data for mice, rabbits and primates were graphed using GraphPad Prism®, Version 5 (GraphPad Software, Inc., San Diego, CA). The area under the plasma concentration–time curve (AUC) in rabbits and primates was calculated using this software from time zero to the time of the last measurable concentration of MeOH or FA in each graph to avoid overestimates of the AUC values. For mouse data, the AUC was calculated using the mean plasma concentration of MeOH or FA at each time point; however, as different animals were euthanized at each time point, the standard deviation of the AUC was determined by the method of Yuan (1993). For both rabbit and primate data, an AUC was determined for individual animals, as each animal was sampled repetitively until 48 or 6 h post-injection, respectively. The clearance (Cl) of MeOH and FA was determined using the equation Cl = dose/AUC. Cl values for mice represent the mean for all animals studied, whereas the Cl data for rabbits and primates represent the elimination of MeOH and FA for each animal.

Statistical analysis. Statistical analysis was performed using GraphPad Prism®, Version 5 (GraphPad Software, Inc.). Differences in MeOH Cl and FA AUC values between mice and rabbits over the complete elimination period were analyzed for significance using the Student's *t*-test. For AUC values in which all 3 species were compared, a one-way analysis of variance (ANOVA) with Tukey's post-hoc test was used. The level of significance was determined to be at $p<0.05$.

Results

Complete time studies

In the time course studies discussed below, plasma concentrations of methanol and FA following saline administration were not detectable, therefore these data were not included in the figures.

MeOH and FA plasma pharmacokinetics following low-dose (0.5 g/kg) MeOH treatment.

Mice. Male CD-1 mice exhibited zero-order (saturation) elimination kinetics of MeOH, with a peak plasma MeOH concentration of approximately 18 mmol/L being achieved between 1 and 4 h post-injection (Figs. 2, and S1). MeOH concentrations were below the level of detection by 8–10 h, leading to a mean clearance (Cl) rate of 3.67 ± 0.46 ml/(min × kg bw) (Fig. 3, Table 1). Additionally, there was an absence of sustained plasma FA accumulation at this dose. A peak in plasma FA occurred at 6 h (0.14 mmol/L) but was undetectable 2 h later, corresponding to an area under the plasma concentration–time curve (AUC (0–24 h)) of 0.53 ± 0.08 (mmol/L) × h.

Rabbits. Male NZW rabbits similarly eliminated MeOH by zero-order kinetics (Figs. 2 and S1). The mean peak plasma MeOH concentration achieved was 24.67 ± 6.74 mmol/L, occurring within 15 min in rabbits 1 and 2, and within 1 h in rabbit 3 (Fig. S2). MeOH was completely eliminated from the plasma by 18 h, corresponding to a mean Cl rate of 1.50 ± 0.26 ml/(min × kg bw) (Fig. 3, Table 1). In each of these rabbits, FA began to accumulate in the plasma within 15 min, with a mean peak concentration of 0.17 mmol/L being achieved between 4 and 6 h. Metabolite accumulation was maintained for 12–30 h, resulting in a mean AUC (0–48 h) of 3.02 ± 1.28 (mmol/L) × h.

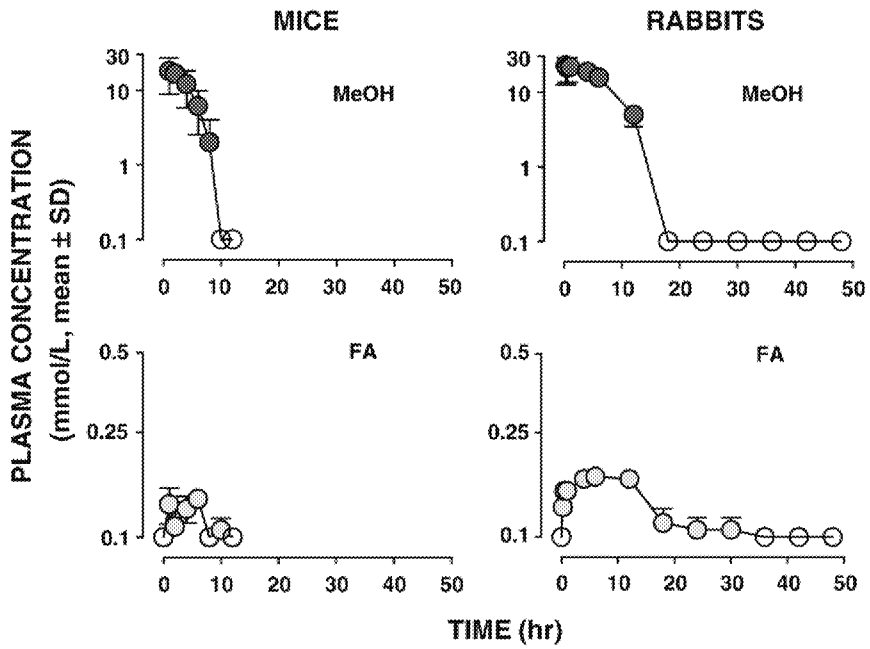

Fig. 2. Mean MeOH and FA pharmacokinetics in male mice and rabbits following a single dose of 0.5 g/kg MeOH, plotted on a semi-logarithmic scale. MeOH was administered through an ip injection as a 20% solution in sterile saline, and plasma samples were analyzed by GC for MeOH or FA concentrations. Saline groups not shown (all not detectable). Each time point represents the mean of 3–6 mice or 3 rabbits for treated groups. Data points with open symbols represent samples with a mean MeOH or FA concentration below the level of detection, and were arbitrarily assigned a value of 0.1 mmol/L.

Species comparison. The MeOH Cl rate in rabbits was less than one-half that in mice at the low dose, although the peak plasma concentrations were not significantly different (Fig. 3). There was a somewhat greater species difference in FA accumulation, with about a 5.7-fold higher FA AUC in rabbits compared to mice. See Table 1 for values and comparisons.

MeOH and FA plasma pharmacokinetics following high-dose (2 g/kg) MeOH treatment.

Mice. In male CD-1 mice, the 2 g/kg dose of MeOH resulted in saturable elimination kinetics (Figs. 4 and S3). A mean peak plasma MeOH concentration of 79 mmol/L was achieved within 1 h, and was 4.4 times greater than that achieved following exposure to the low

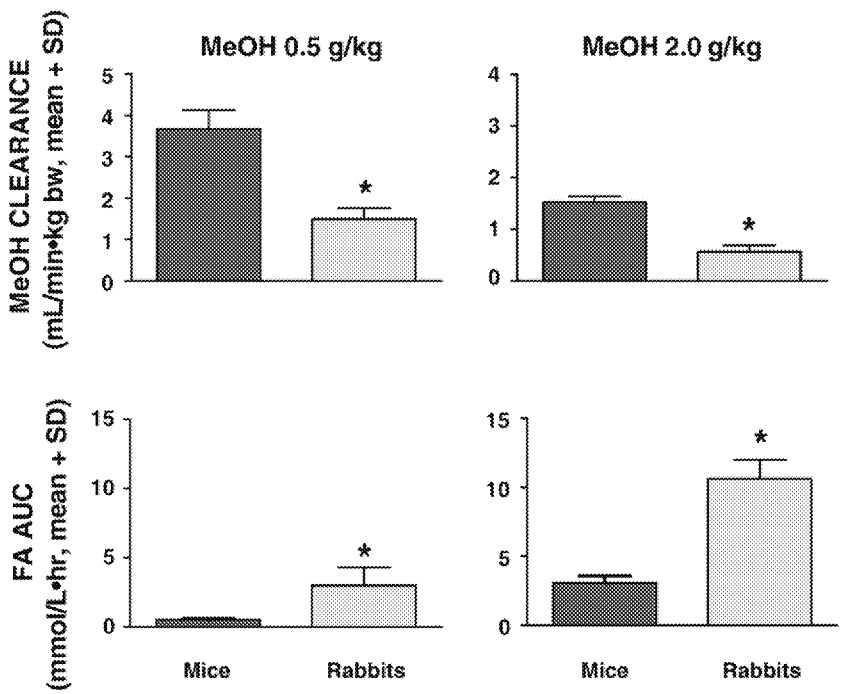

Fig. 3. MeOH clearance (Cl) and FA areas under the plasma concentration–time curve (AUCs) in male mice and rabbits following a single dose of 0.5 or 2 g/kg MeOH. Cl and AUC values were calculated from the MeOH and FA pharmacokinetics curves of animals dosed with 0.5 or 2 g/kg MeOH (20% solution in sterile saline) through an ip injection. Saline groups not shown (all not detectable). Each time point represents the mean of 3–6 mice or 3 rabbits for treated groups. *Indicates measures for rabbit MeOH Cl and FA AUC that were different from the respective mouse values ($p<0.05$).

Table 1
Comparison of rabbits and mice for complete elimination. Studies were carried out over the complete elimination period for rabbits (48 h) and mice (24 h) (see Figs. 2–4, and S1–S4). Data represent the mean ± SD.

Species	Methanol			Formic acid	
	Peak concentration (mmol/L)	AUC (mmol/L × hr)	Cl (ml/min × kg bw)	Peak concentration (mmol/L)	AUC (mmol/L × hr)
High dose (2 g/kg)					
Mouse (0–24 h)	78.86 ± 10.25	697.13 ± 50.47	1.53 ± 0.11	0.31 ± 0.26	3.06 ± 0.54
Rabbit (0–48 h)	113.57 ± 7.15	1892.7 ± 345.3	0.57 ± 0.12	0.28 ± 0.06	10.63 ± 1.35
Low dose (0.5 g/kg)					
Mouse (0–24 h)	18.00 ± 9.10	70.87 ± 8.89	3.67 ± 0.46	0.14 ± 0.26	0.53 ± 0.08
Rabbit (0–48 h)	24.67 ± 6.74	175.3 ± 27.3	1.5 ± 0.26	0.17 ± 0.0	3.02 ± 1.28

dose of MeOH (Fig. 2). This increase is consistent with the 4-fold difference between the low and high doses. The high dose of MeOH resulted in a plasma Cl rate of 1.53 ± 0.11 ml/(min × kg bw) (Fig. 3, Table 1). Even at the higher dose, mice exhibited little FA accumulation, with a mean peak plasma level of 0.3 mmol/L at 8 h, returning to near basal levels within 16–20 h (Fig. 4). Since there was a slight accumulation of FA at 20 h following saline exposure, the final FA AUC (0–24 h) for the 2 g/kg treatment group represents the difference between the MeOH-treated mice and saline controls, and was 3.06 ± 0.54 (mmol/L) × h (Fig. 3, Table 1). This FA AUC (0–24 h) resulting from high-dose MeOH was approximately 5.8-fold higher than that (0.53 (mmol/L) × h) from the low MeOH dose, which in light of the 4-fold difference in doses may suggest saturable FA elimination at the higher dose.

Rabbits. Male NZW rabbits exhibited saturable elimination of MeOH following exposure to the high dose of MeOH (Figs. 4 and S3). A mean peak plasma MeOH concentration of 114 mmol/L was achieved within 15 min. This peak concentration was 5.0 times greater than that achieved following the 0.5 g/kg dose of MeOH, consistent with the 4-fold difference between the high and low doses. The resulting mean plasma MeOH Cl rate following the high dose of MeOH was 0.57 ± 0.12 ml/(min × kg bw) (Fig. 3, Table 1). FA accumulation commenced within 15 min and was maintained for almost 48 h. FA accumulation reached a level 2.8 times greater than that achieved following exposure to the saline control, corresponding to a mean AUC (0–48 h) of 10.63 ± 1.35 (mmol/L) × h (Fig. 3, Table 1). This high-dose AUC reflects a 3.5-fold greater value than that achieved with the low dose, consistent with the 4-fold difference between the two doses.

Species comparison. With high-dose MeOH, the MeOH Cl rate in rabbits was over 60% slower than that achieved in mice (Fig. 3, Table 1). This trend is consistent with the aforementioned species differences in Cl rates observed following the low dose of MeOH exposure. The species difference in FA accumulation observed with low-dose MeOH was substantially enhanced with the high dose, after which the FA AUC in rabbits was nearly 4-fold higher than that in mice. See Table 1 and Fig. 3 for values and comparisons.

Comparison of low- and high-dose studies.

Mice. MeOH Cl in mice treated with high-dose MeOH was about 40% of that following the low dose, while the FA AUC (0–24 h) was 5.8-fold higher (Fig. 3, Table 1).

Rabbits. MeOH Cl in rabbits treated with high-dose MeOH was about 40% of that following the low dose, while the FA AUC (0–48 h) was about 3.5-fold higher (Fig. 3, Table 1).

Six-hour studies

Primates.

Cynomolgus male monkeys treated with 2 g/kg of MeOH or its saline vehicle control were sampled for 6 h, at which time they were

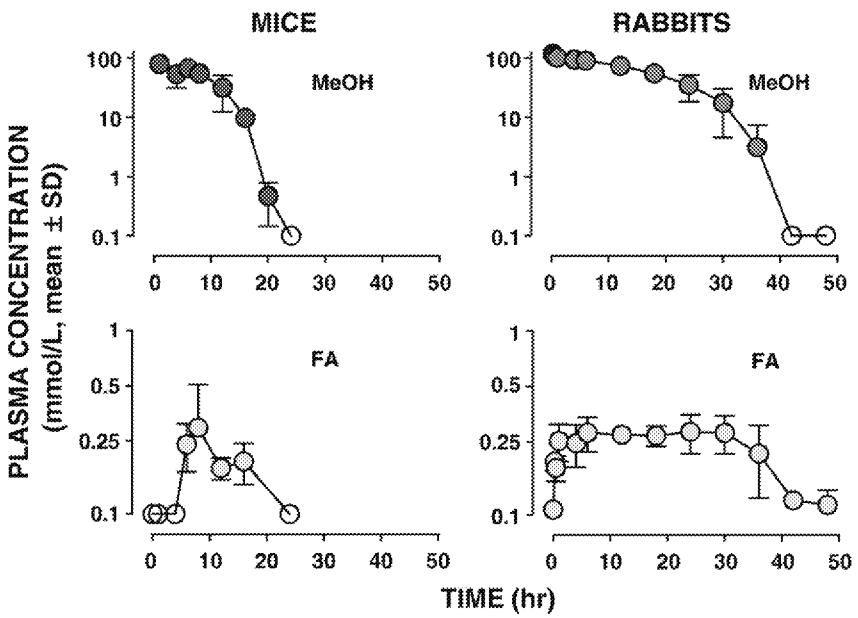

Fig. 4. Mean MeOH and FA pharmacokinetics in male mice and rabbits following a single dose of 2 g/kg MeOH, plotted on a semi-logarithmic scale. MeOH was administered through an ip injection as a 20% solution in sterile saline, and plasma samples were analyzed by GC for MeOH or FA concentrations. Saline groups not shown (all not detectable). Each time point represents the mean of 3–6 mice or 3 rabbits for treated groups. Data points with open symbols represent samples with a mean MeOH or FA concentration below the level of detection, and were arbitrarily assigned a value of 0.1 mmol/L.

euthanized for future molecular studies. All pharmacokinetic values for MeOH and FA accordingly reflected this 6-hour window rather than a complete elimination profile. Within 6 h, MeOH elimination was clearly saturable (Figs. 5, S5 and S6). The mean peak plasma MeOH concentration of 94.49 ± 14.22 mmol/L was achieved within 30 min. FA began to accumulate within 15 min, reaching a relatively substantial concentration of 2.15 ± 0.77 mmol/L at 6 h, at which time it was still increasing.

Species comparison including primates over 6 h.

Plasma concentrations of MeOH over 6 h were similar in primates, rabbits and mice (Fig. 5). The 6-hour plasma MeOH AUC for mice was approximately 1.8 and 1.5-fold lower than the respective AUC values for rabbits and primates (Figs. 6, S7; Table 2). The mean plasma concentration of FA achieved in primates over the 6-hour time course was approximately 11-fold and 7-fold greater than that achieved in mice and rabbits over the same period, respectively (Fig. 5). This difference in primate FA formation is an underestimate, since the concentrations in mice and rabbits, unlike primates, were not still increasing at 6 h. The FA AUC levels achieved within 6 h in primates was about 52-fold and 5-fold higher than the levels in mice and rabbits respectively (Fig. 6). The 6-hour FA AUC in rabbits was 10-fold higher than that in mice.

The 6-hour species difference between mice and rabbits treated with high-dose MeOH (Figs. 5, 6, S7 and S8; Table 2) exceeded that observed in the complete time study for these two species treated with high-dose MeOH, where the complete FA AUC (0–48 h) in rabbits was about 4-fold higher than that in mice (AUC (0–24 h)), and the rabbit MeOH Cl rate was about 37% of that in mice (Figs. 3 and 4; Table 2).

Discussion

Humans primarily use ADH and CYP2E1 to metabolize MeOH to formaldehyde, and subsequently use a capacity-limited, folate-dependent pathway to metabolize FA, which in MeOH poisoning accumulates and is believed to cause the acute MeOH effects of blindness, metabolic acidosis and death (Coon and Koop, 1987; Wallage and Watterson, 2008). In contrast, rodents use catalase to metabolize MeOH, and subsequently use catalase together with a folate-dependent pathway that is not capacity-limited to metabolize FA, which does not accumulate following MeOH overdose (Clary, 2003; Harris et al., 2004). Accordingly, MeOH metabolism in rodents does not reflect that in humans and, as would be expected given the absence of rodent FA accumulation, rodents may not be the most accurate reflection of human risk for the acute toxic effects of MeOH.

Less well understood are the adverse effects of MeOH on the developing embryo and fetus, the mechanisms of which may be quite different from the FA-initiated acute toxicities seen in humans. Although developmental toxicities have not been reported in humans, the potential is assumed largely on the basis of studies in pregnant rodents (Bolon et al., 1994; Harris et al., 2003; Rogers et al., 2004; Degitz et al., 2004). One potential mechanism for developmental abnormalities involves oxidative stress. Even low levels of embryonic and fetal oxidative stress and the formation of ROS have been implicated in the mechanism of developmental toxicities observed in both untreated and xenobiotic-exposed pregnant animal models, and limited evidence suggests a potential role for ROS in the developmental toxicity of MeOH (Farbiszewski et al., 2000; Harris et al., 2004; Parthasarathy et al., 2006). Importantly, ROS can be detoxified by the same catalase enzyme that

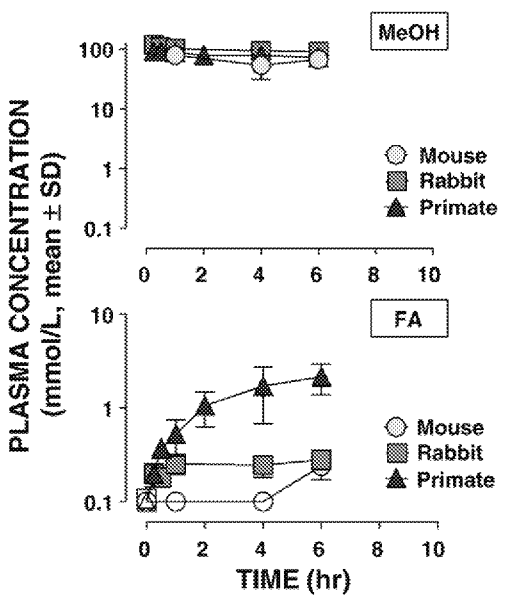

Fig. 5. Mean MeOH and FA pharmacokinetics in male mice, rabbits and primates over a 6-hour time period, following a single dose of 2 g/kg MeOH, plotted on a semi-logarithmic scale. MeOH was administered through an ip injection as a 20% solution in sterile saline, and plasma samples were analyzed by GC (mice and rabbits) or by spectrometry (primates) for MeOH or FA concentrations. A total of 3–6 mice, 3 rabbits or 3 primates were sampled at each time point for treated groups. Saline groups not shown (all not detectable). Data points with open symbols represent samples with a MeOH or FA concentration below the level of detection and were arbitrarily assigned a value of 0.1 mmol/L.

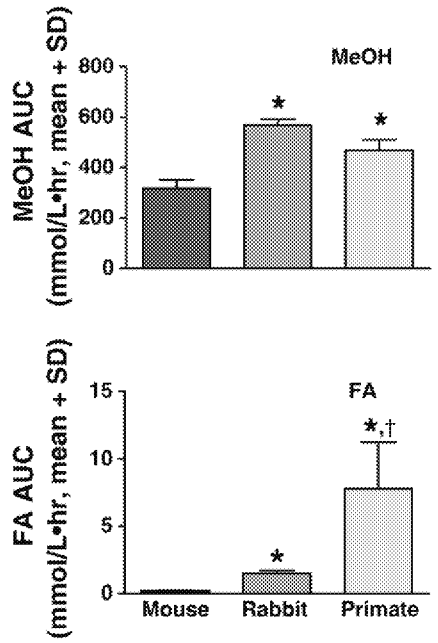

Fig. 6. Six-hour MeOH and FA AUCs in male mice, rabbits and primates following a single dose of 2 g/kg MeOH. AUC values were calculated from the MeOH and FA pharmacokinetics curves of animals dosed with 2 g/kg MeOH (20% solution in sterile saline) through an ip injection. A total of 3–6 mice, 3 rabbits and 3 primates were sampled at each time point for treated groups. Saline groups not shown (all not detectable). *Indicates that 6-hour rabbit and primate MeOH Cl and FA AUC were different from the respective mouse values. †Indicates that the 6-hour FA AUC in primates was different that in rabbits ($p < 0.05$).

Table 2
Comparison of primates, rabbits and mice over 6 h. Studies were carried out for 6 h, at which time primates were euthanized for molecular analyses. (See Figs. 5, 6, and S5–S8). Data represent the mean ± SD.

High dose (2 g/kg)	Methanol		Formic acid	
Species	Peak concentration (mmol/L)	AUC (mmol/L × h)	Peak concentration (mmol/L)	AUC (mmol/L × h)
Mouse	78.86 ± 10.25	316.87 ± 33.28	0.19 ± 0.08	0.15 ± 0.04
Rabbit	113.57 ± 7.15	568.8 ± 23.8	0.28 ± 0.06	1.49 ± 0.23
Primate	94.49 ± 14.22	467.7 ± 43.5	2.15 ± 0.77	7.75 ± 3.48

rodents, but not humans, use to metabolize MeOH. However, it is must be noted that there may be developmental differences in enzyme activity and MeOH metabolism between the embryo and the adult. A recent study suggests that catalase may actually be playing a more influential role than ADH in perinatal human MeOH metabolism (Tran et al., 2007). In light of the profound differences between rodents and humans in metabolic pathways used to metabolize MeOH, and the dual roles of catalase in ROS detoxification and rodent MeOH metabolism, rodent models, while useful in evaluating molecular mechanisms, may not accurately reflect the human risk for MeOH developmental toxicity. We therefore evaluated rabbits as an animal model potentially more similar to humans in their metabolism of MeOH, and if so, possibly more similar to humans in their risk for MeOH developmental toxicity.

We selected the rabbit as an animal model that may potentially more accurately reflect human developmental risk from MeOH for several reasons: (1) one *in vitro* study in hepatic homogenates suggested that the rabbit might be more similar than mice to humans in their use of ADH rather than catalase in MeOH metabolism (Otani, 1978); (2) rabbits and humans, but not rodents, are susceptible to the teratogenic effects of thalidomide, which may be caused in part by ROS formation (Parman et al., 1999; Hansen et al., 2002); and, (3) rats, but not rabbits, are susceptible to glycolic acid-initiated birth defects following exposure to ethylene glycol (Tyl et al., 1993; Carney et al., 2008). Male rabbits and mice were chosen for the metabolism studies herein to avoid potential confounding effects of hormonal variability in females within and between species. Two species comparisons were made, the first over the initial 6 h following high-dose MeOH, which in addition to mice and rabbits included cynomolgus monkeys that were euthanized at this time for molecular studies. The second species comparison for only mice and rabbits was over the full elimination periods for mice (24 h) and rabbits (48 h) following both low- and high-dose MeOH.

The doses used in our studies (2 and 0.5 g/kg) were chosen based upon published reports to deliver tissue concentrations respectively above and below the saturation level for catalase in mice (NEDO, 1986, 1987). However, MeOH elimination following the lower dose still exhibited saturation kinetics in our CD-1 mice, albeit less so than following high-dose MeOH. Since MeOH metabolism is catalyzed by catalase in mice, the low-dose results indicate that a dose lower than that reported elsewhere would be necessary to avoid saturable metabolism in this strain. The saturation kinetic profile for MeOH in rabbits, which appear to use ADH to metabolize MeOH (Otani, 1978), is presumably due to the limited availability of the ADH cofactor NAD^+ (Rang et al., 2003).

As suggested by a previous *in vitro* study using hepatic homogenates (Otani, 1978), our studies herein provide the first *in vivo* evidence that rabbits more closely than mice reflect the human metabolism of MeOH, particularly with respect to FA accumulation. Perhaps most importantly, in the comparison of all 3 species over the first 6 h, the accumulation of FA in primates was 52-fold higher than that in mice. Although rabbits exhibited less FA accumulation than primates, by 6 h rabbits nevertheless exhibited 10-fold higher FA accumulation than mice, more closely reflecting the human profile (Hantson et al., 2005). MeOH AUC over this initial 6-hour period did not differ substantially among species.

Over the complete elimination period, rabbits presented a more predictable dose-dependent pattern of FA accumulation than mice, with a 3.5-fold increase resulting from the 4-fold dose increase going from the low to high dose of MeOH. Mice in contrast accumulated FA to a greater extent, with a 5.8-fold increase following the same increase in MeOH dose. Despite these dose-dependent differences within species, the substantially greater FA accumulation in rabbits compared with mice was about 4-fold higher with high-dose MeOH, and 5.7-fold higher at the low dose. Although both species achieved similar peak concentration levels of FA, the rabbit showed consistently sustained levels over a greater period of time over the course of the study. Conversely, the clearance of MeOH in rabbits was about 40% of that in mice at both the high and low doses of MeOH. Taken together, the lower clearance of MeOH and perhaps most importantly the substantially greater sustained accumulation of FA in rabbits compared to mice indicate that rabbits are substantially more similar than mice to nonhuman primates (our 6-hour study) and humans (Perkins et al., 1995; Clary, 2003) in their metabolism of MeOH. The accumulation of FA may be particularly important in light of the presumed role of this metabolite in the acute toxicities of MeOH, although it is not clear what role FA plays in delayed effects like developmental toxicities (Andrews et al., 1995; Hansen et al., 2005) or cancer, the latter association being controversial due to potentially confounding variables and an absence of corroborating studies (Cruzan, 2009). Additionally, while *in vitro* whole embryo culture studies in mice and rats suggest that formaldehyde may be a potent proximate teratogen mediating MeOH-induced birth defects (Harris et al., 2004; Hansen et al., 2005), the presence of elevated formaldehyde concentrations in tissues and body fluids has not been observed in any species following MeOH exposure (Tephly, 1991), likely due to its transient nature. Formaldehyde has plasma half-lives of approximately 1 and 1.5 min in rats and primates respectively, due to rapid metabolism to FA and/or the formation of DNA and/or protein adducts (Rietbrock, 1965; McMartin et al., 1979; Shaham et al., 1996). During pregnancy, little if any formaldehyde produced in the maternal liver may persist long enough to cross the placenta and enter the embryo. It is not known if toxic amounts of formaldehyde can be produced proximately via the limited activity of embryonic catalase, which at least in mice is less than 5% of maternal activity (Wells et al., 2009).

The values for MeOH clearance and FA accumulation in humans are difficult to determine accurately due to questionable information for such factors as dose or exposure level and time of exposure, as well as the confounding use of hemodialysis therapy as early as possible. In cases of human MeOH poisoning, a wide range of plasma concentrations have been reported, encompassing the values observed herein in all 3 species, with MeOH levels varying from 8 to 154 mM and plasma FA concentrations varying from 0.3 to over 20 mM (Hantson et al., 2005).

In conclusion, rabbits reflected more closely than mice the pharmacokinetic profile of MeOH and FA in primates, the latter of which were similar to the limited published values in humans. This included both the lower clearance of MeOH and greater accumulation of FA, the latter of which is presumed to cause at least the acute toxic effects of MeOH. Whether or not this FA metabolite contributes to delayed MeOH effects like developmental abnormalities remains to be determined, although exogenously added FA is embryotoxic in rodent embryo culture (Harris et al., 2004; Hansen et al., 2005). It is worth noting that the interpretive problem is with the use of mice in the prediction of human risk, since mice, despite the species differences in enzymes and enzymatic activities, can nevertheless be useful in determining molecular mechanisms of teratogens, including alcohols like MeOH and ethanol. It is not known whether the absence of published evidence for delayed outcomes in humans reflects species resistance, the absence of sufficiently high exposures or failure to detect outcomes manifested perhaps weeks, months or years following MeOH exposure. Further studies comparing the developmental effects of MeOH in pregnant rabbits and mice are warranted to determine if rabbits may serve as a more accurate model than mice in predicting human risk with respect to toxicity in the embryo and fetus.

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Appendix A. Supplementary data

Supplementary data are available online, including different graphical representations of data herein, along with more detailed graphical representations of individual data sets (rabbits and primates). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2010.05.009.

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